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Neuropathy Phentoyping Protocols - In Situ Hybridization

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Protocol status: Working
We use this protocol and it's working

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Abstract

Summary:

Phenotyping of Rodents for the Presence of Diabetic Neuropathy
In man, the development of diabetic neuropathy is dependent on both the degree of glycemic control and the duration of diabetes. Diabetic neuropathy is a progressive disorder, with signs and symptoms that parallel the loss of nerve fibers over time. Consequently, assessments of neuropathy in mice are not performed at one time point, but are characterized at multiple time points during a 6 month period of diabetes. The degree of diabetes is evaluated in 2 ways: tail blood glucose measured following a 6 hour fast and glycated hemoglobin levels. The initial degree of neuropathy is screened using the methods discussed below. Detailed measures of neuropathy are employed when the initial screening instruments indicate a profound or unique phenotypic difference. This document contains protocols used by the DiaComp staff to examine and measure diabetic neuropathy at the whole animal, tissue and cellular levels.

This protocol was responsible by *Dr. T. L. Wood*

Diabetic Complication:

Neuropathy

Materials

These reagents and solutions are used for riboprobes both S^{35} and Digoxygenin labeled

Reagents: 2X prehyb and hyb buffer (see hyb/prehyb protocol)
RNase free 4% paraformaldehyde
RNase free ETOH 50, 70, 95%
RNase free 1X PBS
RNase free 20X SSC .2X, .5X, 1X, 2X, 5X
RNase free 20% SDS (Sodium dodecyl sulfate, a.k.a., N-lauroylsarcosine)
DepC water
Triethanolamine (TEA)
Glacial acetic acid
Acetic anhydride (must be dry, store with parafilm around the cap in a desiccant filled container, and
replace every 3 months)
Formamide
1 M dithiothreitol
Rubber cement
RNAse A
Tris buffered saline (TBS)

Supplies: RNase free tips, tubes, cylinders, slide racks and staining dishes
Filter paper
Hyb boxes
21gauge needles
3 ml syringe
Parafilm
Hybriwells

Before start

General Considerations: plastic-staining dishes should be labeled with the solutions they will always contain and should be labeled Day 1 or Day 2. Used this way, they only need to be rinsed with DepC water air-dried and put away. Graduate cylinders should be treated the same way.

Day 1: All solutions, reagents, tips, tubes etc must be RNase FREE!!!

1 List all information concerning slides used, label slides as appropriate

1. Set dry bath at 70ºC and thaw formamide for step 12.

2. Retrieve slides from -80ºC and place directly into a slide rack in a dish containing 4% paraformaldehyde for 2 min (perfusion fixed tissue) or 10 min (fresh frozen tissue)

3. Rinse slides in 1X PBS for 2 min.

4. Rinse in DepC water for 5 min; gently lift rack 1 or 2 times.

5. Dehydrate through EtOH (1 staining dish/dilution, keep for step 10)
    50% 2 min
    70% 2 min
    95% 2 min
    Air-dry for 10 min

6. Rinse slides in fresh DepC, 2 min.

7. Make up fresh TEA: 5.4 ml of TEA + 1.2 ml glacial acetic acid + 400 ml DepC water Mix in a cylinder designated for this solution and this solution only, rinse with DepC water and let dry until next time.

8. Rinse slides in 200 ml of TEA solution, 2 min, remove slide rack, tap on paper towels to remove excess fluid but do not allow slides to dry completely.

9. Place 1 ml of acetic anhydride to a dry staining dish in the hood, place the slide rack in the staining dish and immediately add the other 200 ml of TEA solution.

10. Agitate the rack in the dish vigorously to mix the acetic anhydride and TEA.

11. Incubate 10 min at 22ºC.

12. Rinse in 0.2X SSC for 10 min.

13. Dehydrate through EtOH (1 staining dish/dilution, from step 10)
    50% 2 min
    70% 2 min
    95% 2 min

Remove slides from rack, lay on paper towel
Air-dry for 10 min.

14. If more than one probe is to be used per slide, ring sections with rubber cement. If only one probe is to be used, use hybriwells.

15. Heat denature 2X prehyb buffer at 70ºC for 5 min (dry block) then quickly chill on ice.

16. Set dry block for 85-90ºC for step 14.

17. Mix prehyb 1:1 with formamide. Completely cover each section with enough of this mix to let stand for 1 h at 22ºC.

18. Calculate how much hyb buffer is necessary to cover all of the sections at 50-100 ul per section. Make up the appropriate amount of hyb buffer:
Formamide: 2X hyb, 1:1
1M DTT diluted to 10mM
20% SDS diluted to 0.1%

19. Thaw RNA probe on ice.

20. Calculate volume to add to hyb mix for a specific activity of 4.0 X 10^4 cpm/µl (radioactive probes), or 400 ng/ml (Dig labeled cRNA probes).

21. Heat denature probe at 85-90ºC for no more than 5 min then quickly chill on ice. Do not refreeze any heat denatured probe. Probes may be thawed and refrozen but this should be kept to a minimum. Denatured RNA probe in hyb buffer may be stored at 22ºC until used.

22. Remove prehyb by tilting slides and pipeting off as much as possible. Immediately add appropriate hyb mix to sections.

23. Place sides in a humid box containing 3 MM filter paper soaked in 5X SSC: formamide, 1:1, wrap with plastic (Saran wrap, if probe is fluorescent, wrap with aluminum foil) to prevent evaporation.

24. Hybridize overnight at 60-65ºC with DIG/cRNA. If hyb is radioactive only, 50ºC is ideal.

Day 2: Get these solutions warming before removing slides from oven.

2 200+ ml of 50% fomamide/1X SSC/10mM DTT, in a staining dish 45-50ºC
2 L of 0.2X SSC 50-60ºC, large beaker on warm plate with stir bar.

1. Note any slides on which wells have dried or probes have crossed rubber cement boundaries.

2. Place 2X SSC in a 50 ml conical tube, dip slides then place in rack.

3. Place rack immediately in 50% formamide/1 X SSC/10 mM DTT, cover with parafilm incubate, 30 min.(Store this solution at 4ºC for up to 6 months, refresh DTT every 2 months)

4. Rinse slides in 0.5X SSC for 30 min at 22ºC.

5. Make up 500 ml of RNAse buffer. Volume of RNAse solution, 100 µl X number of sections RNAse solution 0.1 mg/ml RNAse A in RNAse buffer.

6. Remove sections from rack, tap away excess fluid, apply 100 µl RNAse.

7. Incubate for 30 min, @ 22ºC.

8. Tap away RNAse, rinse slides in RNAse buffer 2 X 10 min.

9. Wash slides in 2 L of 0.2X SSC at 50-60ºC for 2 h with gentle stirring.

10. Radioactive in situ only: Dehydrate through ethanol diluted with 300 mM ammonium acetate 2 min, 50, 70, and 95%. Remove rubber cement rings, air dry slides, put on film overnight.

11. For Dig label or double Dig/radioactive skip step 10 and quick dip in TBS.

12. Block for 15 min in Blocking solution. 10% Blocking stock/TBS 1:1 Boehringer.

13. Dilute alkaline phosphatase conjugated anti-Dig 1:500 in the blocking solution.

14. Tap off blocking, add 50-75 µl of anti-Dig.

15. Incubate 1h, 22ºC.

16. Rinse 3 X 3 min in TBS.

17. Place slides in Buffer 3 and add levamisole 1:1000, 10 min.

18. Tap off buffer, add DAKO NBT/BCIP to sections, cover with plastic and incubate 22ºC overnight in the dark.

Day 3:

3 1. Stop color reaction by placing slides in distilled water for 1 min.

2. Rinse 2 X 10 min in 10 mM EDTA/10 mM Tris.

3. Mount coverslips with Permount, for doubles lay down on film prior to dipping.